Virology 401 (2010) 305–313

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Adenovirus 5 and chimeric adenovirus 5/F35 employ distinct B-lymphocyte intracellular trafficking routes that are independent of their cognate cell surface receptor

Mathieu Drouin [a,1], Marie-Pierre Cayer [a,1], Daniel Jung [a,b,*]

[a] Héma-Québec R&D, 1070 avenue des Sciences-de-la-Vie, Quebec City, QC, Canada G1V 5C3
[b] Department of Biochemistry and Microbiology, Laval University, Quebec City, QC, Canada

ARTICLE INFO

Article history:
Received 16 December 2009
Returned to author for revision
3 February 2010
Accepted 1 March 2010
Available online 26 March 2010

Keywords:
Adenovirus
B lymphocytes
Intracellular trafficking

ABSTRACT

Gene transfer applications with adenovirus (Ad) type 5 are limited by its native tropism, hampering their use in several cell types. To address this limitation, several Ad vectors bearing chimeric fiber have been produced to take advantage of the different cellular receptors used by other subgroups of Ads. In this study, we have compared the transduction efficiency of Ad5 and the chimeric Ad5/F35 in primary human B lymphocytes and B-cell lines as a function of the developmental stage. We found that transduction efficiencies of the two Ads differ independently of their targeted cellular receptor but are related to the intracellular localization of the virus. In efficiently transduced cells, Ads were localized in early endosomes or cytosol, whereas in poorly transduced cells they were localized within late endosomes/lysosomes. Finally, we demonstrate that treatment of cells with phosphatase inhibitors known to redirect endocytosis towards caveolae, increased Ad5/F35 transduction efficiency.

Introduction

Adenoviruses (Ads) have the ability to achieve efficient targeting of their genome to the nucleus of infected cells, making them useful tools in gene transfer experiments (Greber and Kasamatsu, 1996; Leopold and Crystal, 2007; Wilson, 1996). Ads can efficiently transduce both proliferating and quiescent cells, and achieve transgene expression within hours (Neering et al., 1996; Nevins et al., 1997). Moreover, Ads do not integrate their DNA into the host cell genome, thus leaving the genetic material of host cells intact. These properties allow reproducible gene expression levels while simultaneously eliminating any undesirable effects related to the site of integration, making Ads attractive tools for the study of transgene effects in transduced cells (Kay et al., 2001). Over 100 different wild-type Ads have been isolated, and about half of them are of human serotype. Among these, Ad subgroup C types 2 and 5 (Ad2 and Ad5) are most commonly used as gene transfer vectors. The Ad fiber knob mediates binding to the coxsackie/adenovirus receptor (CAR) on host cells, allowing subsequent internalization by interacting with $\alpha_V\beta_3$ integrins (Wickham et al., 1993). However, human haematopoietic cells, including lymphocytes, express low levels of CAR and $\alpha_V\beta_3$ integrins (Colin et al., 2004; Huang et al., 1997; McNees et al., 2004), resulting in poor adenoviral transduction efficiencies (Neering et al., 1996; Rebel et al., 2000) for these cells. A new adenoviral vector system (Ad5/F35) was recently developed by substitution of Ad5-based vector fibers with those of a human subgroup B Ad serotype 35 (Havenga et al., 2002; Knaan-Shanzer et al., 2001; Shayakhmetov et al., 2000). This new vector utilizes the ubiquitous CD46 molecule as receptor for entry into host cells (Gaggar et al., 2003). This chimeric Ad5/F35 vector efficiently transduces human haematopoietic progenitor cells (Shayakhmetov et al., 2000), dendritic cells (Rea et al., 2001), primary human T, NK and B lymphocytes (Cayer et al., 2007; Jung et al., 2005; Schroers et al., 2004), primary chronic myeloid leukemia cells and chronic lymphocytic leukemia B cells (Nilsson et al., 2004) and B-type acute lymphoblastic leukemia cells (Yotnda et al., 2004).

Several studies have examined the intracellular trafficking of Ad subgroup C (Leopold and Crystal, 2007); however, most of these were derived from cultured cell lines including HeLa, HEK293 and A549 cells. To our knowledge, only one study has addressed the intracellular fate of Ad5 in lymphoid cells (Colin et al., 2004), and none dealt specifically with normal human B lymphocytes. For Ad subgroup B, including Ad5/F35, only two studies investigated the intracellular trafficking in HeLa and A549 cells (Miyazawa et al., 2001; Shayakhmetov et al., 2003). These reports revealed that cellular trafficking of Ad subgroup C (Ad5) differs from that of subgroup B (Ad7 and Ad/F35), in that Ad5 rapidly escapes early endosomes, whereas Ad7 and Ad5/F35 accumulate in late endosomes. Moreover, it has been demonstrated that in HeLa and A549 cells, the selection of intracellular trafficking routes is determined by the fiber knob and the cellular receptor (Miyazawa et al., 2001; Shayakhmetov et al., 2003).

* Corresponding author. Héma-Québec R&D, 1070 avenue des Sciences-de-la-Vie, Quebec City, QC, Canada G1V 5C3. Fax: +1 418 780 2091.
E-mail address: daniel.jung@hema-quebec.qc.ca (D. Jung).
[1] These authors contributed equally to this manuscript.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.03.003

In this study, we have analyzed the transduction efficiency and intracellular fate of Ad5 in comparison to that of Ad5/F35 in lymphoid cells, including normal human B lymphocytes. We demonstrated that the selection of intracellular trafficking routes is not only determined by the fiber knob domain and the cellular receptor, but mainly by the targeted cell type. Moreover, intracellular entry routes can be modified by exposing cells to phosphatase inhibitors.

Results

Transduction efficiency of Ad5 and Ad5/F35 in various B-lymphocyte lineage cells

We have used two previously constructed Ads, Ad5-EYFP and Ad5/F35-EYFP, containing identical expression cassettes for the EYFP reporter gene (Cayer et al., 2007; Jung et al., 2005) for this study. Ad5-EYFP has an unmodified Ad5 capsid and relies upon CAR as cellular receptor, whereas Ad5/F35 has a modified fiber bearing the knob domain of human serotype 35, thereby modifying its tropism for CD46 as cellular receptor. We have previously demonstrated that Ad5/F35 was substantially more efficient for gene transfer into B lymphocytes than Ad5 (Cayer et al., 2007; Jung et al., 2005). However, we noticed variations in adenovirus-mediated transgene expression among normal human B lymphocytes from different donors and at various B-cell differentiation stages (Jung et al., 2005). We sought to gain further insights into these variations using two adenoviruses with different tropisms.

Eight B-lymphocyte cell lines corresponding to distinct stages of B-lymphopoiesis were investigated: SupB15 (pre-B); Raji, Ramos, Namalwa and Daudi (mature B lymphocytes); L363, RPMI and U266 (plasma cells). Normal human peripheral blood B lymphocytes from three distinct donors were also studied, as well as HEK293 cells, as these cells are highly permissive to both types of adenoviruses. We first analyzed EYFP reporter gene expression after infection with Ad5-EYFP or Ad5/F35-EYFP at a MOI of 500. As expected 100% of HEK293 cells were EYFP-positive following infection with either adenovirus (Fig. 1), confirming the high permissivity of this cell line for Ad5 (Colin et al., 2004) and Ad5/F35 (Cayer et al., 2007). In contrast, normal human B lymphocytes were poorly transduced by Ad5, whereas Ad5/F35 was able to transduce these cells to some extent (Fig. 1). However, we observed noticeable variations between samples from different donors for both adenoviruses. For instance, B lymphocytes from one donor were moderately permissive, with 45% and 20% EYFP-positive cells when transduced with Ad5/F35 and Ad5, respectively, whereas B cells from the other two donors were not as permissive, with about 20% and 2–8% EYFP-positive cells after transduction with Ad5/F35 and Ad5, respectively. Broader variations in transduction efficiency of both adenoviruses were observed in cell lines of B-cell origin, as shown in Fig. 1. Cell lines of plasma cell origin were efficiently transduced with both adenoviruses, especially RPMI and U266 cells (near 100% EYFP-positive cells); L363, the least efficiently transduced cell among plasma cell lines, still gave 64% and 88% EYFP-positive cells after transduction with Ad5 and Ad5/F35, respectively. Among mature B-cell lines, Daudi was the least efficiently transduced, with around 20% EYFP-positive cells after infection with either adenovirus, whereas Raji was strongly transduced by Ad5 (63% EYFP-positive cells) but much less by Ad5/F35 (17% EYFP-positive cells). Ramos and Namalwa cells were poorly transduced by Ad5 (~20% EYFP-positive cells) and moderately transduced by Ad5/F35 (~30–40% EYFP-positive cells). Pre-B-cell lines (SupB15) were hardly transduced by Ad5 (2% EYFP-positive cells); in contrast, these cells were fairly well transduced by Ad5/F35, with 58% EYFP-positive cells after infection. These results confirmed our previous observation that Ad5/F35 were more efficient to transduce lymphoid cells than Ad5. Collectively, these results clearly demonstrate that there are donor- and differentiation stage-dependent variations in adenovirus transduction efficiency among cells from the B-lymphocyte lineage.

Analyses of the level of EYFP expression in transduced cells by QPCR confirmed the results described above (data not shown). Ad5-EYFP infection led to a 100- to 100 000-fold increase in EYFP transcript in U266 cells compared the other B-lymphoid cell lines. However, similar levels of EYFP transcript were detected in HEK293 and U266 cells, suggesting that Ad5 transduced these cell lines with comparable efficiencies. Differences in EYFP transcript levels among the various B-cell models used were less pronounced when using Ad5/F35-EYFP, with 50- to 1000-fold higher transcript levels in U266 cells compared to the other B-cell models.

Variations in surface CAR expression among B-lymphoid cell types

It has been reported that Ad5 requires CAR for cellular binding and $\alpha_v\beta_3$ integrin for internalization (Wickham et al., 1993). Notably, B-lymphoid cells do not express this integrin (Colin et al., 2004; Huang et al., 1997; Rebel et al., 2000), suggesting that this membrane protein is not essential for Ad5 internalization in B lymphocytes. We have also observed a very low percentage ($\leq$2%) of $\alpha_v\beta_3$-positive cells

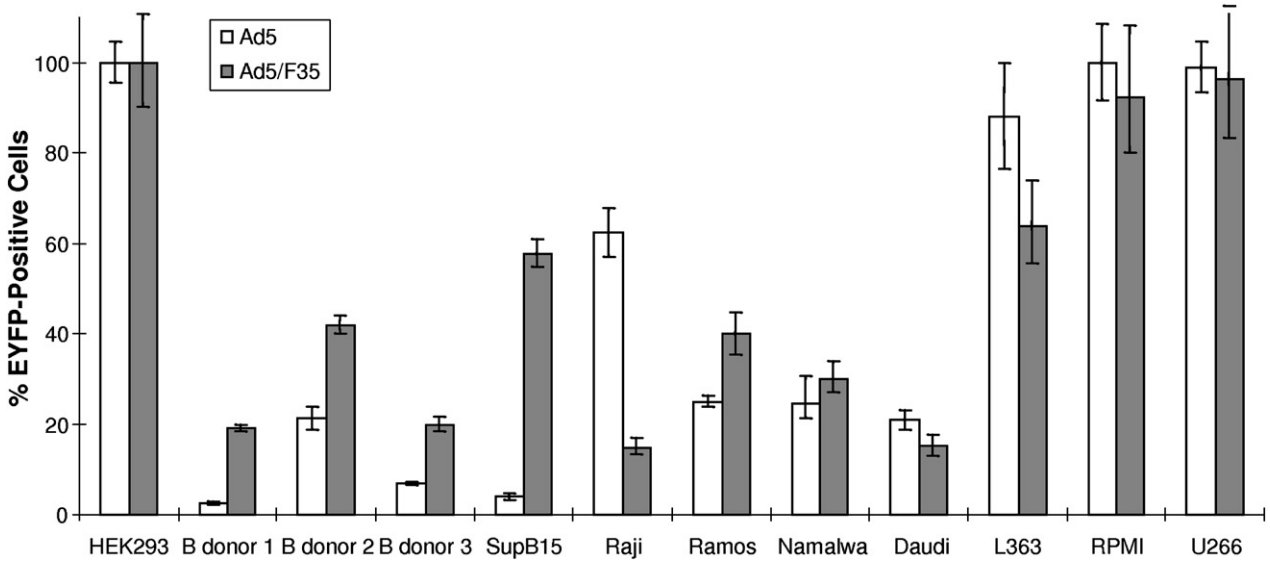

Fig. 1. Comparison of adenoviral transduction efficiencies between the chimeric Ad5/F35-EYFP vector and the parental Ad5-EYFP vector in normal human B lymphocytes, HEK293 and lymphoid cells. Cells were infected with adenovectors at a MOI of 500 for one hour, as described in Materials and methods. EYFP expression was analyzed 48 h post-transduction by flow cytometry. Results represent the mean of four independent experiments, with errors bars corresponding to standard deviations.

within populations of normal B lymphocytes and B-cell lines compared to HEK 293 cells (data not shown). Additionally, it has been shown that Ad5/F35 requires only CD46 for binding and internalization (Gaggar et al., 2003; Shayakhmetov et al., 2000). Therefore, variations in adenovirus transduction efficiency could be attributed to the differential expression of their respective cellular receptors. We sought to gain further insights into these differences in transduction efficiency by analyzing CAR and CD46 protein levels in lymphoid cell lines by flow cytometry. Our results shown in Fig. 2 indicate that except for HEK293, Daudi and Raji, less than 50% of the cells from the tested lymphoid lines express CAR. Surprisingly, U266 cells show a very low percentage of positive cells (15%), yet these cells appeared fully permissive to Ad5 transduction in previous experiments. Conversely, Daudi cells are non-permissive to Ad5 transduction, despite a higher percentage of CARpositive cells (85%) than HEK293 (78%). Furthermore, Raji cells were fairly permissive to Ad5 transduction, with 70% EYFP-positive cells upon infection, even though they expressed lower levels of CAR than Daudi cells. Clearly, alternative pathways for Ad5 infection of lymphoid cell lines exist, and CAR may not be necessarily involved.

In contrast to CAR, CD46 was expressed by the vast majority of the cells of lymphoid origin that we have analyzed, including normal B lymphocytes (Fig. 2), yet the permissivity of these cells to Ad5/F35 was low, except for plasma cell lines. Thus, as for Ad5, it appears that Ad5/F35 transduction efficiency does not correlate with CD46 expression. Collectively, these observations suggest that adenovirus transduction efficiency is not solely dependent on the adenoviral fiber protein and cellular receptors as previously stated (Shayakhmetov et al., 2003), but is also related to the identity of the infected cell.

Binding and uptake of Ad5 and Ad5/F35 by lymphoid cells

To further investigate the differences in transduction efficiency of Ad5 and Ad5/F35 in lymphoid cells, we have performed qualitative analysis of their respective binding and uptake. Adenoviruses were labeled with carboxynaphthofluorescein, and binding and uptake analyses were performed on normal B lymphocytes, HEK293 and cell lines that showed diverse transduction efficiencies (SupB15, Daudi and U266). Cells were incubated in the presence of labeled adenoviruses. An aliquot of cells was analyzed by flow cytometry to determine adenovirus binding after a 60-minute incubation at 4 °C; a second aliquot was further incubated for 30 min at 37 °C to allow adenovirus uptake. Cells were trypsinized to remove cell surface-bound adenovirus particles and uptake was quantified by flow cytometry analysis. As shown in Fig. 3, with lymphoid cell lines, percent binding and uptake for both adenoviruses were similar. However, with normal human B lymphocytes, we observe less fluorescein-positive cells in uptake experiments than in binding experiments for both types of viruses. This result indicates that not all attached adenovirus particles enter cells. Notably, for all cell types analyzed, Ad5/F35 virus had higher binding and uptake than Ad5. Moreover, among lymphoid cell lines, Daudi cells displayed the highest adenovirus binding and uptake, yet they were poorly permissive to Ad5 and Ad5/F35 transduction. Conversely, U266 cells, which are fully permissive to adenovirus transduction, showed lower virus binding and uptake. These results suggest that binding and internalization are not the sole determinants of transduction efficiency, and points to intracellular processing of viral particles as an additional parameter influencing transduction.

Intracellular localization of Ad5 and Ad5/F35 in lymphoid cells

It is well-documented that adenoviruses enter cells *via* endocytic pathways (see (Sieczkarski and Whittaker, 2002) for review). When entering cells by endocytosis, adenoviruses first localize in early endosomes before experiencing one of three possible fates: i) escape early endosomes and move to the nucleus; ii) move to recycling endosomes to be excreted; or iii) move to late endosomes and either escape to the nucleus or remain within lysosomes where they eventually get degraded. Early endosomes and cytoplasm have a pH of 6.5/7, whereas pH values for late endosomes and lysosomes are in the range of 5.5/4.5. We have exploited these differences to analyze intracellular trafficking of adenoviruses. Ad5 and Ad5/F35 were labeled with the pH-sensitive fluorophore carboxynaphthofluorescein (CNF), which requires neutral to alkaline pH (6.5/8) for maximal

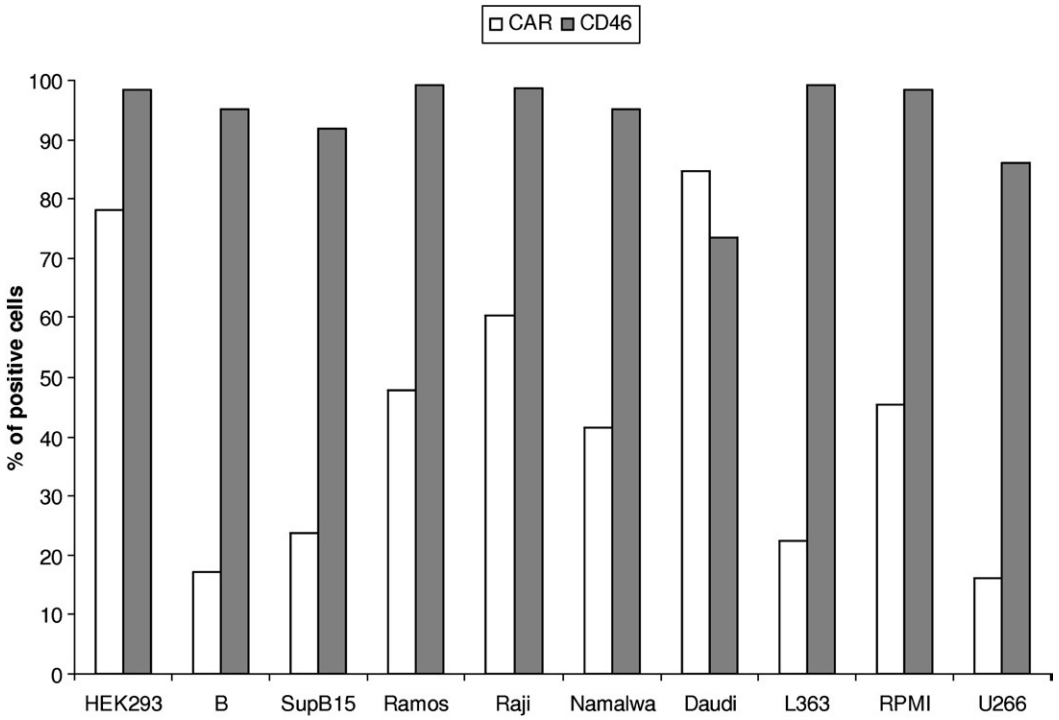

Fig. 2. Analyses of CAR and CD46 expression in normal human B lymphocytes, HEK293 and lymphoid cells. CAR and CD46 expression was analyzed by flow cytometry. Results are expressed as percentage of positive cells for CAR (grey bars) and CD46 (white bars).

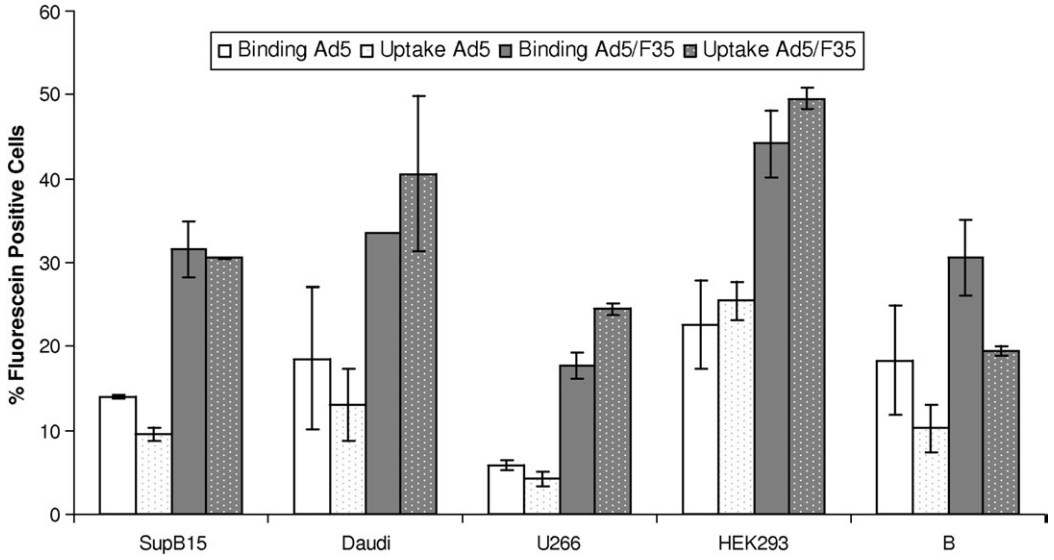

Fig. 3. Binding and uptake of CNF-labeled Ad5 and Ad5/F35 in lymphoid cells. For binding experiments, cells were incubated with CNF-labeled adenoviruses at a MOI of 100 for one hour on ice, as described in Materials and methods. Quantification of cell-attached viral particles by flow cytometry was performed after fixation with 1% paraformaldehyde. For the determination of the internalized fraction of CNF-labeled viruses, cells were resuspended in 100 µl of adhesion buffer after binding experiments, and incubated at 37 °C for 30 min. Cells were diluted threefold with cold 0.05% trypsin–0.5 mM EDTA and incubated at 37 °C for an additional 10 min. Cells were pelleted and resuspended in one ml ice-cold PBS and fluorescence was analyzed by flow cytometry as described above. Results represent the mean percentage of fluorescein-positive cells from three independent experiments, with errors bars corresponding to standard deviations.

fluorescence (Thomas et al., 1979), or pHrodo™, for which fluorescence emission requires acid pH (lower than 6) and is non-fluorescent outside the cell (Harvey et al., 2008; Miksa et al., 2009; Moore et al., 2008). Virus labeling with these dyes does not alter infectivity (data not shown). Next, labeled adenoviruses were allowed to bind to cells for 40 min at 4 °C and uptake was initiated by transferring cells incubated with viruses at 37 °C. Analyses were performed after 50 min of incubation at 37 °C, at which time the majority of Ad5 particles localized to the perinuclear space (Leopold et al., 1998; Miyazawa et al., 2001). For Ad5, the results of Fig. 4a indicate that fluorescence could only be detected in HEK293, SupB15 and U266 cells infected with CNF-labeled Ad5. These results clearly demonstrate that Ad5 particles do not migrate to late endosomes, but rather stay in early endosomes or escape rapidly to the cytoplasm as previously suggested by Shayakhmetov et al. (2003). In contrast, faint fluorescence was detected in B lymphocytes infected with CNF-labeled Ad5, whereas strong fluorescence could be detected in cells infected with pHrodo-labeled Ad5. The latter finding suggests that in normal B lymphocytes, Ad5 localize mainly within late endosomes or lysosomes. In Daudi cells, fluorescence could be detected in cells infected with either labeled adenoviruses. Taken together, these results suggest that the selection of intracellular trafficking routes taken by internalized adenoviral particles depends largely on the identity of the infected cell.

Results obtained with labeled Ad5/F35, shown in Fig. 4b, differ markedly from those obtained with labeled Ad5. For HEK293, SupB15 and U266 cells, fluorescence could be detected following infection with CNF- and pHrodo-labeled Ad5/F35. However, fluorescence was much brighter with CNF-labeled Ad5/F35, suggesting that most Ad5/F35 remained in and/or escaped early endosomes towards either the nucleus or the extracellular space, rather than being redirected towards later endosomes/lysosomes as previously described for Ad5/F35 in HeLa cells (Shayakhmetov et al., 2003). In contrast, normal B lymphocytes infected with CNF- or pHrodo-labeled Ad5/F35 showed comparable fluorescence emission, whereas in Daudi cells, fluorescence could only be detected after infection with pHrodo-labeled Ad5/F35. These results clearly demonstrate that the intracellular trafficking of Ad5/F35 is cell-specific.

Finally, we failed to detect intracellular fluorescence from labeled adenoviruses when internalization times exceeding 50 min were applied. However, the EYFP reporter gene product could be detected as soon as two hours after beginning the internalization incubation, suggesting that adenovirus transduction, when occurring, is very rapid (data not shown). Moreover, when internalization times ranging from 15 to 30 min were applied, only CNF-labeled Ads could be detected by fluorescence emission analyses in all cell types tested (data not shown). This suggests that Ads localize within early endosomes in the first 30 min that follow internalization, before eventually migrating to late endosomes.

An alternative entry route for Ad5/F35

Inefficient Ad5/F35 transduction of B lymphocytes is a major challenge for testing gene function in these cells. One way to circumvent a possible limitation of transduction would be to bypass the endosome/lysosome entry route. Several viruses such as SV40, murine polyomavirus, picornavirus, echovirus 1, and respiratory syncytial virus (Gilbert and Benjamin, 2000; Kartenbeck et al., 1989; Marjomaki et al., 2002; Pelkmans et al., 2001; Pelkmans et al., 2002; Richterova et al., 2001; Werling et al., 1999) use caveolae as a route of cellular entry, bypassing the traditional endosome/lysosome pathway (Pelkmans et al., 2001). Internalizations involving caveolae proceed more slowly, and the resulting vesicles do not become acidified; however this process is not constitutive, and only occurs upon cell stimulation (Thomsen et al., 2002). Several studies have shown that caveolae endocytosis can be increased by treating cells with phosphatase inhibitors such as okadaic acid (Tagawa et al., 2005; Thomsen et al., 2002). We therefore determined the transduction efficiency of Ad5/F35-EYFP in SupB15 and Daudi cells upon okadaic acid treatment. As shown in Fig. 5a, the percentage of EYFP-positive SupB15 cells increased from 65% in untreated cells to 85% and 95% in cells treated with 50 nM and 25 nM okadaic acid, respectively; no increases in transduction efficiency were observed in SupB15 cells treated with 100 nM okadaic acid. The same compound had similar effects on Daudi cells: the percentage of EYFP-positive cells increased from 5% in untreated cells to 25%, 24% and 45% in cells treated with 100 nM, 50 nM and 25 nM okadaic acid, respectively. We have also analyzed the effects of okadaic acid on Ad5/F35-EYFP transduction efficiency in normal human B lymphocytes. As shown in Fig. 5b,

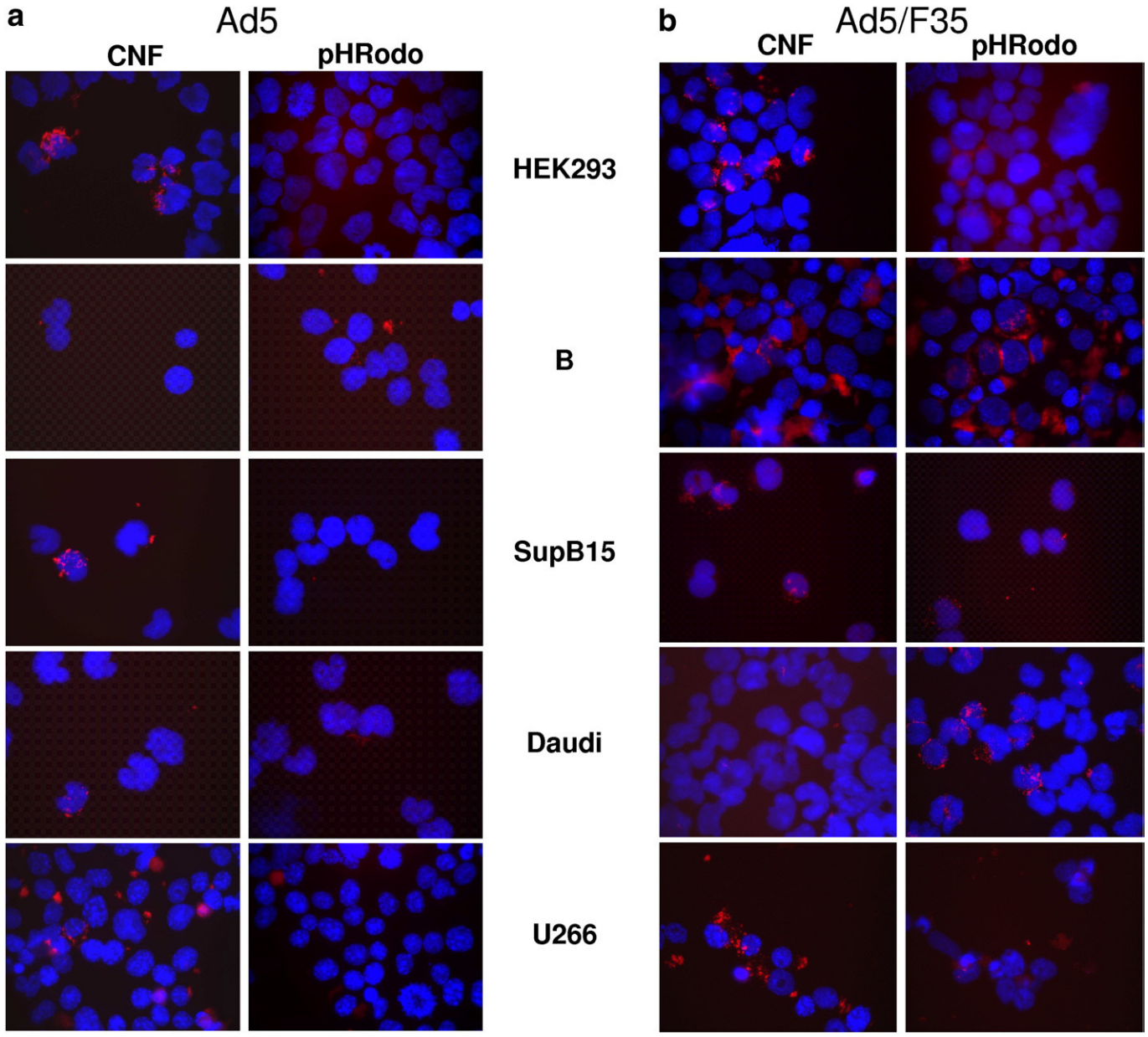

Fig. 4. Intracellular trafficking of adenoviruses in lymphoid cells. Intracellular localization of CNF- or pHrodo-labeled adenoviruses (MOI = 100) after a 40-minute binding incubation at 4 °C and a 50-minute internalization incubation at 37 °C, analyzed by fluorescence microscopy. a) Ad5. b) Ad5/F35. The representative fields are shown at a magnification of ×1000.

following treatment of B lymphocytes with 25 and 30 nM okadaic acid, the percentage of EYFP-positive cells increased from 35% to 65%, respectively. Transduction efficiency declined (45% to 55%) with higher concentrations of okadaic acid. Finally, we have observed that concentrations of okadaic acid ≥50 nM have cytotoxic effects on B lymphocytes and B-cell lines (data not shown).

Taken together, our results clearly demonstrate that Ad5/F35 transduction efficiency can be significantly increased in lymphoid cells by the use of phosphatase inhibitors, such as okadaic acid.

Discussion

We have shown that transduction efficiency of Ad5 and Ad5/F35, which utilize distinct cellular receptors, varies among human B lymphocyte from different donors and lineages. Previous studies have shown that transduction efficiency could be modulated by intracellular trafficking, which is influenced by the cellular receptor used by the adenovirus and the structure of the adenoviral fiber and knob (Miyazawa et al., 1999, 2001; Shayakhmetov et al., 2003). However, these studies were performed on only two cell lines, HeLa and A549 cells. Recently, Colin et al. (2004) demonstrated than lymphoid cells are differentially permissive to Ad5; unfortunately, these authors did not provide any data for Ad5/F35. Our study, which includes several cell lines from lymphoid origin as well as normal human B lymphocytes, demonstrates that there is no direct correlation between transduction efficiency and cellular receptor expression for both types of viruses. For instance, only 15% of U266 cells express CAR, yet these cells are highly permissive to Ad5 transduction, as are HEK293 cells where CAR is expressed in 80% of the cells. In contrast, 85% of Daudi cells are positive for CAR, but this cell line is not permissive to Ad5 transduction. Furthermore, Raji cells were fairly permissive to Ad5, with 70% of the cells EYFP-positive after infection, yet CAR expression in Raji is lower than in Daudi cells. Moreover, while CD46, the cellular receptor for Ad5/F35, is ubiquitously expressed, some cells remain poorly permissive to this adenovirus. Collectively, these observations indicate that the structure and/or level of expression of the cognate adenovirus

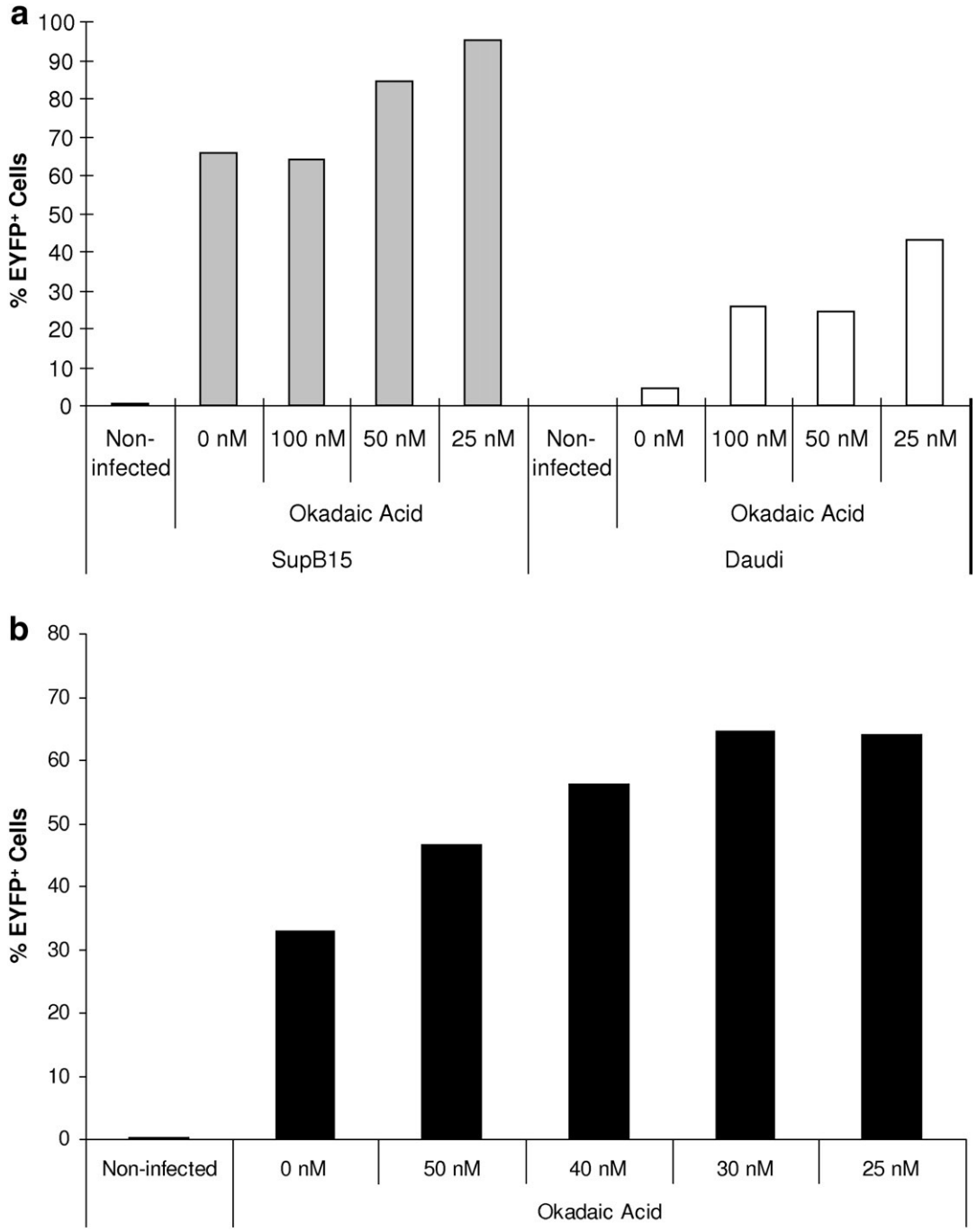

Fig. 5. Ad5/F35-EYFP transduction efficiencies in lymphoid cells and normal human B lymphocytes. Cells were treated for four hours with okadaic acid at the indicated concentrations and infected with Ad5/F35-EYFP at a MOI of 500 for one hour, as described in Materials and methods. EYFP expression was analyzed 48 h post-transduction by flow cytometry. a) SupB 15 and Daudi cell lines. b) Normal human B lymphocytes. Results are expressed in percentage of EYFP-positive cells.

receptor is not predictive of transduction efficiency. Interestingly, McNees et al. (2004) have reported a study of adenovirus gene expression in T-lymphoid cell lines that reached conclusions similar to those presented herein. The authors showed that restoring expression of CAR failed to make naturally restrictive T cells more permissive for adenovirus in spite of the apparent delivery of virus into the cell. As for our observations, one could speculate that Ads might be trapped in late endosome/lysosomes in T-lymphoid cells as well.

An intriguing point that we noticed is the high transduction efficiency of Ad5 in U266, L363 and RPMI cells, given that most of these cells lack CAR expression. However, Colin et al. (2005, 2004) previously showed that Ad5 efficiently transduces U266 cells using a lipid raft/caveolae endocytic route; the same pathway might also be working in the two other plasma cell lines. In any event, the possibility that Ad5 bypasses the traditional endosome/lysosome entry route opens new avenues to increase Ad5 transduction efficiency in presumably non-permissive cells.

One of the major points of the present study is that adenovirus transduction efficiency depends largely on cell type-specific intracellular trafficking routes. We have used pH dependent fluorophores to analyze the subcellular localization of labeled adenovirus particles. It has previously been shown that this labeling procedure does not alter the natural trafficking of adenovirus (Colin et al., 2004; Miyazawa et al., 1999, 2001; Shayakhmetov et al., 2003), allowing pHrodo and carboxynaphthofluorescein to become powerful tools for the study of endocytosis and phagocytosis (Harvey et al., 2008; Miksa et al., 2009; Moore et al., 2008; Thomas et al., 1979). Using these fluorophores, we

have demonstrated the presence of distinct, cell-specific intracellular trafficking routes for Ad5 and Ad5/F35 viruses, and that these pathways operate independently of the presence or absence of specific cellular receptors. In cells with low transduction efficiency, most Ad5 or Ad5/F35 particles are directed towards late endosomes/lysosomes. In contrast, in cells that are highly permissive to adenovirus transduction, most Ad5 or Ad5/F35 particles localize within early endosomes or cytosol. Notably, Ad5/F35 could be detected in both types of endosomes in normal human B lymphocytes, in which an intermediate level of transduction efficiency is observed with this virus. Our results suggest that the intracellular localization of internalized adenovirus is a key determinant of the transduction efficiency. Following their migration to late endosomes and lysosomes, adenoviruses are exposed to a low pH, protease-rich environment that might eventually lead to their degradation. The lack of adenoviral transgene expression might result from such a lysosomal degradation. However, one cannot exclude the possibility that some adenoviruses exploit the low pH environment to their advantage and escape late endosomes, as previously described for Ad5/F35 and adenoviruses from subgroup B (Miyazawa et al., 1999, 2001; Shayakhmetov et al., 2003). This possibility could explain the faint transgene expression observed in Daudi cells and normal human B lymphocytes. Alternatively, low or absent transgene expression could also be attributed to the endocytic recycling pathway that would extrude adenovirus particles outside the cells.

Factors determining which intracellular trafficking routes are effective in a given cell remain to be investigated. We observed that HEK293 and plasma cell lines (L363, RPMI and U266), all highly active in proteins synthesis and specifically immunoglobulins for plasma cell lines, are also highly permissive for Ad5 and Ad5/F35. In contrast, Ad5 and Ad5/F35 transduction efficiency was low or intermediate in normal human B lymphocytes, SupB15 (pre-B), Raji, Ramos, Namalwa and Daudi (mature B lymphocytes); these cells are generally less active than plasma cells and show comparatively low protein synthesis activity. We hypothesize that the protein synthesis activity of a given cell might dictate the intracellular trafficking of adenovirus. In cells with low protein synthesis activity, adenoviruses might be trapped in late endosomes/lysosomes, whereas in cells actively synthesizing proteins, adenoviruses might be able to escape early endosomes and gain access to the cytosol and eventually the nucleus where transcription of viral genes takes place. Alternatively, the lipid raft/caveolae endocytic route might allow for an efficient transduction as well.

Another important finding of our study is that Ad5/F35 transduction efficiency can be significantly increased in lymphoid cells following phosphatase inhibitors treatment. Such treatment has previously been shown to stimulate caveolae endocytosis (Tagawa et al., 2005; Thomsen et al., 2002). This suggest that Ad5/F35 transduction efficiency could be significantly increased in lymphoid cells through stimulation of caveolae endocytosis, circumventing the traditional endosome/lysosome entry route to use instead caveolae to cross the plasma membrane. These preliminary results will require further investigations to confirm that Ad5/F35 effectively enter cells by this pathway. However, it is reasonable to assume that Ad5/F35 could also use this entry pathway following cell stimulation, as has been reported for Ad5 in U266 cells (Colin et al., 2005). Additionally, our results suggest that adenoviruses do not strictly rely on a single canonical cellular receptor to enter the cell. This represents an interesting avenue for the construction of new chimeric adenoviruses as recently reported by Rogee et al. (2007), in that it might allow to take advantage of non-classical endocytic pathways compensating for a low transduction efficiency in certain cell types.

Materials and methods

Cell lines

SupB15 (pre-B acute lymphoblastic leukemia), Raji (Burkitt's lymphoma), Ramos (Burkitt's lymphoma), Namalwa (Burkitt's lymphoma), Daudi (Burkitt's lymphoma), L363 (plasma cell leukemia), RPMI (multiple myeloma), U266 (multiple myeloma) and HEK293 (human embryonic kidney) cell lines were obtained from ATCC (Manassas, MD, USA). HEK293A (QBI-293A), purchased from Q.BIOgene (Carlsbad, CA, USA), contain adenoviral genes E1 and E3 for *in vitro* adenovirus production. SupB15, Namalwa, and Ramos cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). Daudi, Raji, U266, RPMI and L363 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI) (Invitrogen) supplemented with 15% FBS (Invitrogen). HEK293 and HEK293A were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 5% FBS. Cell density and viability were monitored daily by Trypan blue dye exclusion using a haemacytometer. All cell lines used were mycoplasma-free.

Culture of human peripheral blood B lymphocytes

Blood samples were obtained from healthy individuals after informed consent, and peripheral blood mononuclear cells were prepared by density centrifugation over Ficoll-Paque (GE Healthcare Biosciences Inc., Baie D'Urfé, QC, Canada). B lymphocytes were purified by negative selection using the StemSep CD19 mixture according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). Purified human B lymphocytes were >95% $CD19^+$, as determined by flow cytometry. B lymphocytes were cultured at 37 °C and 10% CO_2 in a humidified atmosphere for five days in the presence of SCM-7 membrane preparations expressing CD154 (ratio: 340×10^6 CD154 molecules/25 000 B cells), as previously described (Cayer et al., 2007), in IMDM supplemented with 10% Ultra-Low IgG FBS, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, antibiotics, (all from Invitrogen), 100 U/ml IL-4 (R&D Systems, Minneapolis, MN, USA), 50 U/ml IL-2 and 25 U/ml IL-10 (both from PeproTech, Rocky Hill, NJ, USA). These specific CD154 signal strength and cytokine cocktail are required for activation and proliferation of human B lymphocytes (Fecteau and Néron, 2003; Neron et al., 2005). Cell counts and viability were evaluated in triplicate by Trypan blue dye exclusion. Cultured B lymphocytes were always >96% $CD19^+$, and unless specified otherwise, viability was >85%.

Production of adenoviral vectors

Ad5-EYFP and Ad5/F35-EYFP vectors were generated by *in vivo* recombination in *E. coli* BJ5183 bacteria between pAdenoVator transfer plasmids and pAdEasy-1 or pAdEasy-1/F35 adenoviral genomes using the AdenoVator™ vector system (Qbiogene, Inc., Carlsbad, CA, USA). Transfer plamids containing CMVi were purchased from Qbiogene. The EYFP gene from pIRES-EYFP (Clontech, Palo Alto, CA, USA) was cloned in the transfer plasmids described above. Recombinant adenovirus vectors were transfected into QBI-293A cells (Qbiogene) using LipoFectamine 2000 (Invitrogen) and recombinant viruses were plaque-purified. Viruses were amplified, purified and stored at −80 °C, as previously described (Cayer et al., 2007). Viral stock titers the ranged between 3×10^8 and 1×10^9 infectious units/µl.

Adenoviral infection

Adenoviral infection of B lymphocytes was performed as previously described (Jung et al., 2005). Briefly, cells were washed with culture medium and resuspended at a density of 1×10^7 cells/ml in 100 µl of culture medium containing virus at a multiplicity of infection (MOI) of 500. After one hour at 37 °C, cells were transferred into 24-well culture plates and diluted to a final volume of one ml with their respective culture medium. Cells were further incubated for 48 h after transduction before harvesting and analysis.

Flow cytometry analysis

Allophycocyanin-conjugated anti-CD19, FITC-conjugated anti-CD46, PE-conjugated anti-CAR, and allophycocyanin-, PE-, and FITC-conjugated isotype controls were used in staining procedures. All antibodies (Abs) were murine IgG_1 monoclonal Abs obtained from BD Biosciences (Mountain View, CA, USA) or Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) (anti-CAR). All stainings were performed using one µg of each Ab for 1×10^6 cells at 4 °C. Cells were fixed with 2% paraformaldehyde. In all analyses, >95% of the cells were double negative when using isotype-matched control Abs. Regions containing dead cells were gated using 7-amino-actinomycin D staining, following the manufacturer's instructions (BD Biosciences). The proportion of $EYFP^+$ $7\text{-}AAD^-$ cells was determined by flow cytometry (FACSCalibur, Becton Dickinson, Palo Alto, CA, USA). A minimum of 10000 gated cells were acquired, and analyses were done with the CellQuest Pro software and FCSExpress software (De Novo Software, Thornhill, ON, Canada).

Real-time quantitative PCR

Total RNA was isolated from 10^6 cells using the High Pure RNA isolation kit (Roche Diagnostics, Laval, QC, Canada). One µg of RNA was used to prepare cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo-dT. Quantitative PCR (QPCR) reactions were performed with the Perfecta SYBRgreen fast mix (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions, on a Mx3005P QPCR system (Stratagene). For each time point, the average of three replicate reactions was calculated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control for normalization. The following primer sequences were used: *GADPH* F (5′ ATG CAA CGG ATT TGG TC 3′); *GADPH* R (5′ TCT CGC TCC TGG AAG ATG GTG 3′); *EYFP* F (5′ACG TAA ACG GCC ACA AGT TC 3′); *EYFP* R (5′ AAG TCG TGC TGC TTC ATG TG 3′). Primer specificity was verified by melting curve analysis. The comparative Ct method was used to calculate relative mRNA expression by normalizing with a housekeeping gene control (GAPDH) and EYFP levels were expressed relative to levels of the same transcript in infected U266 cells.

Labeling of adenoviruses with pH-sensitive fluorophore

Two fluorescent dyes were used. Carboxynaphthofluorescein (CNF) is characterized by a maximum fluorescence emission at pH above 8, and very low emission between pH 6 and 5, and finally no emission at pH lower than 5. pHrodo™ fluorescence emission can only be observed at pH lower than 6, and is very strong at pH around 4.

Adenovirus capsids were labeled with CNF or pHrodo, according to the manufacturer's protocol using a dye molecule/VP ratio of 2.68×10^6 and 2.37×10^6 respectively for CNF and pHrodo. Briefly, coupling with 5,6-carboxynaphthofluorescein succinimidyl ester (Invitrogen) and pHrodo™ succinimidyl ester (Invitrogen) was performed in 0.1 M sodium carbonate, pH 8.3. 5,6-carboxynaphthofluorescein succinimidyl ester (400 µg/ml) or pHrodo™ succinimidyl ester (400 µg/ml) were added to 12.5×10^9 VP and further incubated for 30 min at room temperature. The reaction mixture was then transferred to a dialysis chamber (Slide-A-Lyzer, 10000 MW cutoff, Pierce, Rockford, IL, USA) and dialyzed against a solution of 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl and 10% glycerol at 4 °C to remove unincorporated chemicals.

Attachment and internalization assays

These assays are based on a published protocol (Shayakhmetov et al., 2000). For attachment studies, 1×10^6 cells were incubated for one hour on ice with CNF-labeled adenoviruses at a MOI of 100 in 100 µl of ice-cold adhesion buffer made up of culture medium supplemented with 2 mM $MgCl_2$, 1% bovine serum albumin, and 20 mM HEPES. Cells were pelleted by centrifugation for 5 min at 500 ×*g* and washed three times with one ml ice-cold PBS. Quantification of labeled viral particles was performed by flow cytometry after cell fixation with 1% paraformaldehyde. To determine the fraction of CNF-labeled viruses that were internalized, cells were infected as described above but after the last wash, cells were resuspended in 100 µl of adhesion buffer and incubated for 30 min at 37 °C. Cells were diluted threefold with cold 0.05% trypsin–0.5 mM EDTA solution and incubated at 37 °C for an additional 10 min. This treatment removes 99% of cell-bound viruses (Shayakhmetov et al., 2003). Finally, cells were pelleted and resuspended in one ml ice-cold PBS and fluorescence was analyzed by flow cytometry as described above.

Analysis of adenoviruses trafficking

75000 cells were incubated in 100 µl with labeled adenovirus (MOI = 500) for 40 min at 4 °C, followed by an additional 50-minute incubation at 37 °C. Cells were then centrifuged at 60 ×*g* for 5 min onto microscope slides using the cytospin technique. Cells were washed three times with PBS, fixed with 4% formaldehyde for 15 min, and washed three times in permeabilization buffer (PBS/0.1% Triton X-100). Cells were blocked with PBS/0.1% Triton X-100/1% BSA for 10 min and incubated with DAPI for 20 min. Finally, slides were washed three times with PBS/0.1% Triton X-100 and mounted in Prolong Glod (Invitrogen). Slides were analyzed with a Nikon Eclipse TE200-S fluorescence microscope.

Cell treatment with phosphatase inhibitors

Cells at a density of 5×10^5/ml were treated for 4 h with okadaic acid (Sigma) at concentrations specified in the Results section. Cells were then washed three times with PBS-glucose and infected with Ad5/F35-EYFP as described above. 48 h postinfection, cells were analyzed by fluorescence microscopy and flow cytometry.

Acknowledgments

We are grateful to Jean-François Leblanc for critical reading and comments of the manuscript. Finally, we are thankful to all the participants of this study, and to Claudine Côté for coordination of blood sample collection.

This work was financially supported in part by a grant from the Bayer/Talecris/Canadian Blood Services/Héma-Québec Partnership Fund to DJ.

References

Cayer, M.P., Drouin, M., Sea, S.P., Forest, A., Cote, S., Simard, C., Boyer, L., Jacques, A., Pineault, N., Jung, D., 2007. Comparison of promoter activities for efficient expression into human B cells and haematopoietic progenitors with adenovirus Ad5/F35. J. Immunol. Methods 322, 118–127.

Colin, M., Renaut, L., Mailly, L., D'Halluin, J.C., 2004. Factors involved in the sensitivity of different hematopoietic cell lines to infection by subgroup C adenovirus: implication for gene therapy of human lymphocytic malignancies. Virology 320, 23–39.

Colin, M., Mailly, L., Rogee, S., D'Halluin, J.C., 2005. Efficient species C HAdV infectivity in plasmocytic cell lines using a clathrin-independent lipid raft/caveola endocytic route. Mol. Ther. 11, 224–236.

Fecteau, J.F., Néron, S., 2003. CD40 stimulation of human peripheral B lymphocytes: distinct response from naïve and memory cells. J. Immunol. 171, 4621–4629.

Gaggar, A., Shayakhmetov, D.M., Lieber, A., 2003. CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9, 1408–1412.

Gilbert, J.M., Benjamin, T.L., 2000. Early steps of polyomavirus entry into cells. J. Virol. 74, 8582–8588.

Greber, U.F., Kasamatsu, H., 1996. Nuclear targeting of SV40 and adenovirus. Trends Cell Biol. 6, 189–195.

Harvey, B.P., Quan, T.E., Rudenga, B.J., Roman, R.M., Craft, J., Mamula, M.J., 2008. Editing antigen presentation: antigen transfer between human B lymphocytes and macrophages mediated by class A scavenger receptors. J. Immunol. 181, 4043–4051.

Havenga, M.J., Lemckert, A.A., Ophorst, O.J., van Meijer, M., Germeraad, W.T., Grimbergen, J., van Den Doel, M.A., Vogels, R., van Deutekom, J., Janson, A.A., de Bruijn, J.D., Uytdehaag, F., Quax, P.H., Logtenberg, T., Mehtali, M., Bout, A., 2002. Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. J. Virol. 76, 4612–4620.

Huang, S., Stupack, D., Mathias, P., Wang, Y., Nemerow, G., 1997. Growth arrest of Epstein–Barr virus immortalized B lymphocytes by adenovirus-delivered ribozymes. Proc. Natl. Acad. Sci. U. S. A. 94, 8156–8161.
Jung, D., Neron, S., Drouin, M., Jacques, A., 2005. Efficient gene transfer into normal human B lymphocytes with the chimeric adenoviral vector Ad5/F35. J. Immunol. Methods 304, 78–87.
Kartenbeck, J., Stukenbrok, H., Helenius, A., 1989. Endocytosis of simian virus 40 into the endoplasmic reticulum. J. Cell Biol. 109, 2721–2729.
Kay, M.A., Glorioso, J.C., Naldini, L., 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat. Med. 7, 33–40.
Knaan-Shanzer, S., Van Der Velde, I., Havenga, M.J., Lemckert, A.A., De Vries, A.A., Valerio, D., 2001. Highly efficient targeted transduction of undifferentiated human hematopoietic cells by adenoviral vectors displaying fiber knobs of subgroup B. Hum. Gene Ther. 12, 1989–2005.
Leopold, P.L., Crystal, R.G., 2007. Intracellular trafficking of adenovirus: many means to many ends. Adv. Drug Deliv. Rev. 59, 810–821.
Leopold, P.L., Ferris, B., Grinberg, I., Worgall, S., Hackett, N.R., Crystal, R.G., 1998. Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum Gene Ther. 9, 367–378.
Marjomaki, V., Pietiainen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypia, T., Heino, J., 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76, 1856–1865.
McNees, A.L., Mahr, J.A., Ornelles, D., Gooding, L.R., 2004. Postinternalization inhibition of adenovirus gene expression and infectious virus production in human T-cell lines. J. Virol. 78, 6955–6966.
Miksa, M., Komura, H., Wu, R., Shah, K.G., Wang, P., 2009. A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. J. Immunol. Methods 342, 71–77.
Miyazawa, N., Leopold, P.L., Hackett, N.R., Ferris, B., Worgall, S., Falck-Pedersen, E., Crystal, R.G., 1999. Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. J. Virol. 73, 6056–6065.
Miyazawa, N., Crystal, R.G., Leopold, P.L., 2001. Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. J. Virol. 75, 1387–1400.
Moore, N.M., Sheppard, C.L., Barbour, T.R., Sakiyama-Elbert, S.E., 2008. The effect of endosomal escape peptides on in vitro gene delivery of polyethylene glycol-based vehicles. J. Gene Med. 10, 1134–1149.
Neering, S.J., Hardy, S.F., Minamoto, D., Spratt, S.K., Jordan, C.T., 1996. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood 88, 1147–1155.
Neron, S., Racine, C., Roy, A., Guerin, M., 2005. Differential responses of human B-lymphocyte subpopulations to graded levels of CD40–CD154 interaction. Immunology 116, 454–463.
Nevins, J.R., DeGregori, J., Jakoi, L., Leone, G., 1997. Functional analysis of E2F transcription factor. Methods Enzymol. 283, 205–219.
Nilsson, M., Ljungberg, J., Richter, J., Kiefer, T., Magnusson, M., Lieber, A., Widegren, B., Karlsson, S., Fan, X., 2004. Development of an adenoviral vector system with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells. J. Gene Med. 6, 631–641.
Pelkmans, L., Kartenbeck, J., Helenius, A., 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat. Cell Biol. 3, 473–483.
Pelkmans, L., Puntener, D., Helenius, A., 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science 296, 535–539.
Rea, D., Havenga, M.J., van Den Assem, M., Sutmuller, R.P., Lemckert, A., Hoeben, R.C., Bout, A., Melief, C.J., Offringa, R., 2001. Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells. J. Immunol. 166, 5236–5244.
Rebel, V.I., Hartnett, S., Denham, J., Chan, M., Finberg, R., Sieff, C.A., 2000. Maturation and lineage-specific expression of the coxsackie and adenovirus receptor in hemato-poietic cells. Stem Cells 18, 176–182.
Richterova, Z., Liebl, D., Horak, M., Palkova, Z., Stokrova, J., Hozak, P., Korb, J., Forstova, J., 2001. Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. J. Virol. 75, 10880–10891.
Rogee, S., Grellier, E., Bernard, C., Loyens, A., Beauvillain, J.C., D'Halluin, J.C., Colin, M., 2007. Intracellular trafficking of a fiber-modified adenovirus using lipid raft/caveolae endocytosis. Mol. Ther. 15, 1963–1972.
Schroers, R., Hildebrandt, Y., Hasenkamp, J., Glass, B., Lieber, A., Wulf, G., Piesche, M., 2004. Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35 chimeric adenoviral vectors. Exp. Hematol. 32, 536–546.
Shayakhmetov, D.M., Papayannopoulou, T., Stamatoyannopoulos, G., Lieber, A., 2000. Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. J Virol. 74, 2567–2583.
Shayakhmetov, D.M., Li, Z.Y., Ternovoi, V., Gaggar, A., Gharwan, H., Lieber, A., 2003. The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. J. Virol. 77, 3712–3723.
Sieczkarski, S.B., Whittaker, G.R., 2002. Dissecting virus entry via endocytosis. J.Gen. Virol. 83, 1535–1545.
Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., Helenius, A., 2005. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J. Cell Biol. 170, 769–779.
Thomas, J.A., Buchsbaum, R.N., Zimniak, A., Racker, E., 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18, 2210–2218.
Thomsen, P., Roepstorff, K., Stahlhut, M., van Deurs, B., 2002. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol. Biol. Cell 13, 238–250.
Werling, D., Hope, J.C., Chaplin, P., Collins, R.A., Taylor, G., Howard, C.J., 1999. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. J. Leukoc. Biol. 66, 50–58.
Wickham, T.J., Mathias, P., Cheresh, D.A., Nemerow, G.R., 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell. 73, 309–319.
Wilson, J.M., 1996. Adenoviruses as gene-delivery vehicles. N. Engl. J. Med. 334, 1185–1187.
Yotnda, P., Zompeta, C., Heslop, H.E., Andreeff, M., Brenner, M.K., Marini, F., 2004. Comparison of the efficiency of transduction of leukemic cells by fiber-modified adenoviruses. Hum. Gene Ther. 15, 1229–1242.